

Docket No.: 28113/39467A  
(PATENT)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

---

In re Patent Application of:  
Kari Alitalo et al.

Application No.: 10/567,630

Confirmation No.: 2853

Filed: May 30, 2006

Art Unit: 1634

For: MATERIALS AND METHODS FOR  
COLORECTAL CANCER SCREENING,  
DIAGNOSIS, AND THERAPY

---

Examiner: S. T. Kapushoc

**DECLARATION PURSUANT TO 37 CFR §1.131**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

We, Tatiana Petrova, Ph.D., Kari Alitalo, Ph.D., and Antti Nykanen, Ph.D.,  
hereby declare and state as follows:

1. We are the co-inventors of the invention presently claimed in the above-identified patent application. We understand that the United States Patent and Trademark Office (PTO) has rejected one or more claims of the application based, at least in part, on the disclosure of Parr and Jiang, "Quantitative analysis of lymphangiogenic markers in human colorectal cancer," *International Journal of Oncology*, 23: 533-539 (2003), which the PTO says was publicly available as of 17 July 2003. We understand that this reference is cited as prior art to the extent that it describes the invention "before the invention thereof by the applicant for patent." We make this declaration to provide evidence to PTO that the inventors had performed research and possessed at least as much information relevant to the invention as shown in Parr (2003), before the July 2003 publication date of Parr (2003).

2. Attached hereto as Exhibit A and incorporated by reference is an invention disclosure document that we prepared before July 2003, summarizing work that we

had performed in our laboratory in Helsinki, Finland (a WTO country) and conclusions that we had drawn from our work. We communicated this invention disclosure document to US patent counsel at Marshall Gerstein & Borun before July 2003 and received facsimile confirmation that it was received and understood.

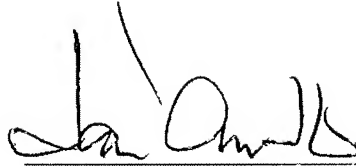
3. The PTO cited Parr (2003) for its teaching of elevated Prox-1 mRNA in colon cancer as compared to normal colon mucosa. Our invention disclosure document describes that we measured Prox-1 mRNA in colorectal tumor samples and matched normal controls, and that elevated Prox-1 mRNA was measured in 35/53 colorectal tumor samples measured (70%). In contrast, no increase in Prox-1 was measured in several other types of tumors that we tested. We also describe experiments where we analyzed the types of cells that were expressing Prox-1, and experiments that showed elevated Prox-1 expression in the SW480 colon cancer line. We provided a hypothesis for how Prox-1 over-expression can contribute to colon cancer progression, and conceived that Prox-1 targeted therapy can be beneficial in the treatment of this type of cancer.

4. Attached as Exhibit B and incorporated by reference is a draft manuscript that we communicated to the owner of the patent application, Licentia Ltd. of Helsinki, Finland, and that Licentia further communicated to US patent counsel at Marshall Gerstein & Borun before July 2003. The manuscript describes and expands upon the experiments summarized in the invention disclosure attached as Exhibit A. In addition to the experiments described in the invention disclosure, the manuscript describes experiments in which we used siRNA to suppress Prox-1 in a highly malignant subclone of the SW480 colon cancer cell line. Suppression of Prox-1 resulted in growth arrest. These findings further supported our idea that suppression of Prox-1 may prove beneficial in the treatment of colon cancer.

5. We each hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: October 19, 2010

By



**Kari Alitalo, Ph.D.**

Date: October \_\_, 2010

By

**Tatiana Petrova, Ph.D.**

Date: October \_\_, 2010

By

**Antti Nykanen, Ph.D.**

Date: October \_\_\_, 2010

By \_\_\_\_\_  
**Kari Alitalo, Ph.D.**

Date: October \_\_19\_\_, 2010

By \_\_\_\_\_  
  
**Tatiana Petrova, Ph.D.**

Date: October \_\_\_, 2010

By \_\_\_\_\_  
**Antti Nykanen, Ph.D.**

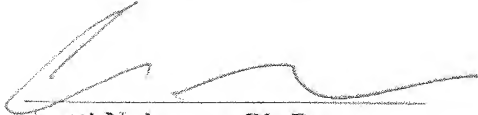
Date: October \_\_\_, 2010

By \_\_\_\_\_  
Kari Alitalo, Ph.D.

Date: October \_\_\_, 2010

By \_\_\_\_\_  
Tatiana Petrova, Ph.D.

Date: October 20, 2010

By  \_\_\_\_\_  
Antti Nykanen, Ph.D.

## Exhibit A

## **Transcription factor Prox-1 is overexpressed in colorectal cancer.**

Tatiana V. Petrova<sup>1</sup>, Lauri Aaltonen<sup>2</sup> and Kari Alitalo<sup>1</sup>

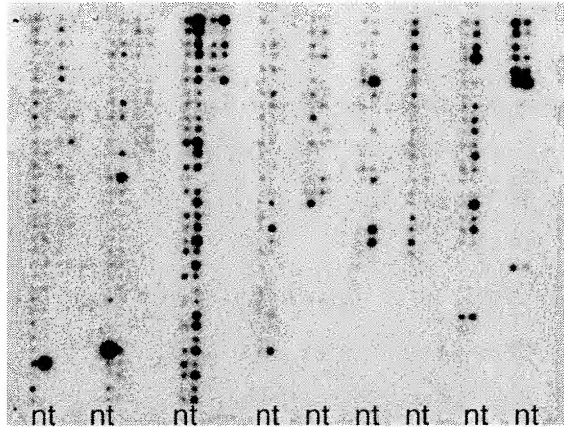
<sup>1,2</sup>Molecular/Cancer Biology Program and <sup>1</sup>Ludwig Institute for Cancer Research, Haartman Institute and Helsinki University Hospital, Biomedicum Helsinki, University of Helsinki, FIN-00014 Helsinki, Finland.

Transcription factor Prox-1 is widely expressed during development and it is essential for growth of lymphatic vessels, migration of hepatocytes and lens fiber elongation. We found that mRNA for Prox-1 was elevated in 35/53 colorectal tumor samples examined (70%) in comparison to matched normal colon samples. In contrast, no increase of Prox-1 was observed in breast, uterus, stomach, lung, ovary, kidney or thyroid cancers. Levels of Prox-1 did not correlated with levels of lymphatic endothelial marker LYVE-1 suggesting that lymphatic vessels are not the major sites of Prox-1 expression (Fig.1). Immunofluorescent staining using affinity purified antibody against human Prox-1 revealed staining of isolated cells in normal colonic epithelium, which likely correspond to neuroendocrine cells (Fig2 A-I), whereas all cells were positive for Prox-1 in tumor sample (Fig. 2D-I). Prox-1 is also strongly expressed in subpopulation of SW480 colon cancer line (Fig. 2J-L). Our previous results demonstrated that adenoviral gene transfer of Prox-1 in a range of primary human cells up-regulate expression of genes involved in cell cycle progression such as cyclin E1 and E2, PCNA, DHFR and thymidine kinase<sup>1</sup>. We hypothesize that Prox-1 overexpression can contribute to colon cancer progression by increasing proliferative potential or survival of tumor cells, and that anti-Prox-1 therapy can be beneficial in the treatment of this type of cancer.

1. Petrova TV, Makinen T, Makela TP, Saarela J, Virtanen I, Ferrell RE, Finegold DN, Kerjaschki D, Yla-Herttuala S, Alitalo K EMBO J. 2002 Sep 2;21(17):4593-9.

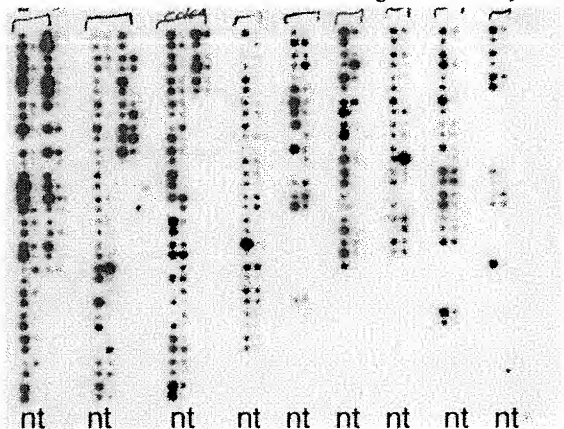
breast uterus colon stom ov lung kid rect thyroid

Prox-1



breast uterus colon stom ov lung kid rect thyroid

LYVE-1



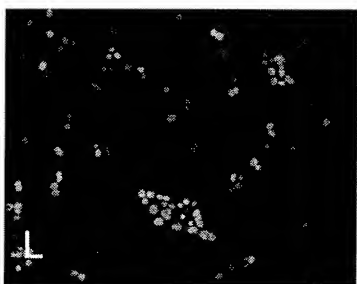
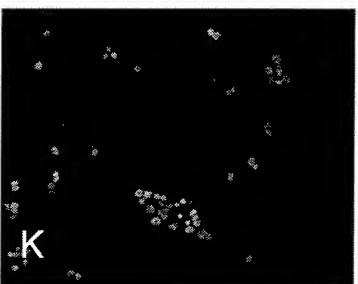
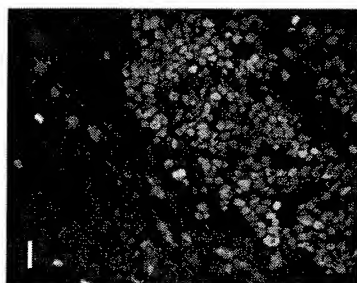
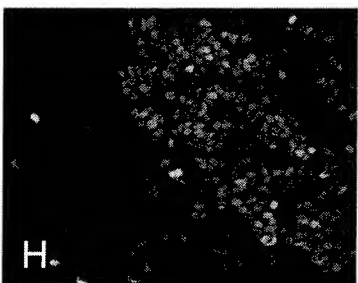
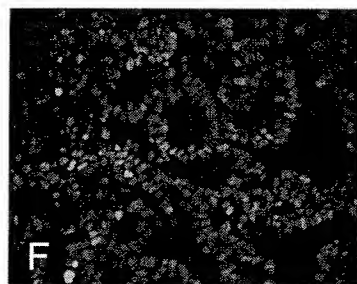
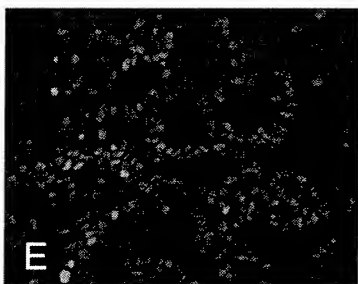
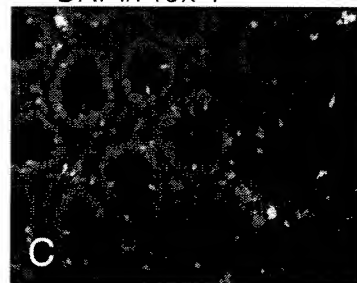
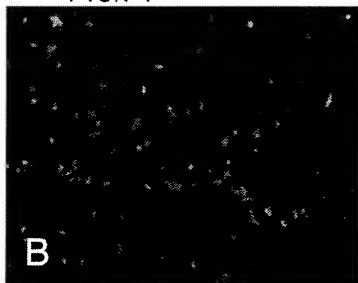
n=normal  
t=tumor



DAPI

Prox-1

DAPI/Prox-1



## Exhibit B

**Overexpressed homeobox transcription factor Prox-1 counteracts a Notch-dependent epithelial differentiation program in colorectal cancer**

Tatiana V. Petrova<sup>1</sup>, Antti Nykanen<sup>1</sup>, and Kari Alitalo<sup>1</sup>

<sup>1</sup> Molecular/Cancer Biology Laboratory, Ludwig Institute for Cancer Research and <sup>2</sup>Human Tumorigenesis Group

Haartman Institute and Helsinki University Hospital

Biomedicum Helsinki Molecular and Cancer Biology Program

Biomedicum Helsinki

University of Helsinki

00014 Helsinki

Finland

## **Abstract**

Transcription factor Prox-1 is essential during development for lens fiber elongation, hepatocyte migration and the sprouting of lymphatic vessels from embryonic veins. Here we describe findings which implicate Prox-1 in colorectal cancer. Prox-1 mRNA was increased in 68% of colorectal tumors in comparison with matched normal colon samples (n=53). In contrast, no increase of Prox-1 was observed in breast, uterine, stomach, lung, ovarian, kidney or thyroid cancers. Immunohistochemical analysis of the tumors and premalignant lesions of colon confirmed increased Prox-1 protein levels in a variable proportion of the colorectal adenoma and adenocarcinoma cells, whereas the normal colonic mucosa showed staining only in neuroendocrine cells and epithelial cells at the base of the crypts. In order to investigate a potential mechanism by which Prox1 can participate in colon cancer progression, we silenced Prox1 in highly malignant subclone of colon adenocarcinoma SW480 using small interfering RNAs. Suppression of Prox1 resulted in growth arrest, shift of the transcriptional program towards a more differentiated epithelial-like phenotype through the up-regulation of Notch signaling. On the other hand, Prox-1 upregulation in differentiated subclones of SW480 cells via an adenovirus decreased the expression of Notch2 and its ligand jagged1. Our study identify for the first time Prox1 as an important regulator of colon cancer progression and suggest that it may control differentiation of colonic epithelium by regulating the expression of key components of Notch pathway. Our findings suggest that suppression of Prox1 or induction of Notch pathway through the expression of its signaling components, such as jagged1, Notch receptors or transcription factor Hey2, may prove beneficial in the treatment of colon premalignant and cancer lesions.

## Results.

### *Prox-1 is overexpressed in colorectal cancer.*

We investigated the expression of Prox-1 mRNA in human cancers using a cancer gene profiling array filter, which contains cDNAs from about 250 human cancers and corresponding normal control tissues. Prox-1 mRNA was significantly increased in 35 out of 53 samples of colorectal cancers. In contrast, only rarely or no increase was seen in samples from breast, uterine, lung, kidney, ovarian or thyroid tumors (**Fig. 1A**). Interestingly, expression of Prox-1 was low or absent in all kidney cancer samples present. Prox-1 is a marker for lymphatic vessels, which are abundant both in normal colon and around colonic carcinomas (Hajime). We therefore hybridized the filter to the probe for the lymphatic endothelial hyaluronan receptor LYVE-1. Unlike Prox-1, LYVE-1 levels were higher in the normal samples, suggesting that the increased expression cannot be attributed to the lymphatic vessels (Fig. 1B).

We further investigated the expression of Prox-1 in colon cancers using affinity purified antibodies raised against Prox-1 homeobox and prospero domains, which are conserved between the mouse and human proteins. These antibodies specifically recognized Prox-1 in transiently transfected 293T cells. Staining of a panel of mouse tissues and E12.5 and E17.5 embryos revealed specific nuclear staining for Prox-1 in the previously reported sites of expression such as in lymphatic vessels, lens fiber cells and in a subset of neurons in the neural tube (supplementary data). Staining of twelve human adenomas and nine human colorectal carcinomas and adjacent normal mucosa revealed increased expression of Prox-1 in eleven adenoma samples and in six tumor samples (**Fig. 2A-F**). Increased Prox-1 staining was observed in all cells in 10 adenoma samples and in two tumor samples, whereas in the others a heterogeneous expression of Prox-1 occurred. In one tumor sample, no specific staining for Prox-1 was seen, while strong expression was observed in intratumoral lymphatic vessels (data not shown).

In the normal colonic mucosa, Prox-1 was strongly expressed in a subset of epithelial cells, a subset of which were positive for the pan-neuroendocrine marker chromogranin A (Fig. 2I-L). In addition, we observed a weaker but significant Prox-1 expression in the bottom of the crypts below the cell proliferation zone identified by staining for the Ki-67 antigen (Fig. 2M-O and Ref). The location of Prox1 positive cells at the base of the crypts corresponds to the position of the intestinal stem cells (Ref.).

*Prox-1 is expressed in round but not in adherent subclones of the SW480 colon adenocarcinoma cell line.*

No Prox-1 expression was seen in the majority of tumor cell lines studied (n=25, see supplementary material). However Prox-1 mRNA was present in hepatocellular carcinoma cell line HepG2 and the colon carcinoma cell line SW480 (**Fig. 3A** and not shown). Immunofluorescent staining of Prox-1 revealed strong expression in all HepG2 cells (not shown), whereas only a subset of SW480 cells was Prox-1 positive. Double immunofluorescent staining for Prox-1 and for  $\beta$ -catenin or for the F-actin marker phalloidin demonstrated that Prox-1 expression is restricted to weakly adherent round SW480 cells which did not display focal adhesions or actin stress fibers, and that Prox-1 was almost absent from the adherent cells (arrows in Fig. 3B-G). The existence of two subtypes of cells in the SW480 cultures was reported previously (refs). Interestingly, the SW480R (round) cells displayed anchorage independent growth *in vitro* and highly malignant phenotype *in vivo*, whereas the SW480A (adherent) cells did not grow well in soft agar and formed small and well differentiated tumors when implanted into nude mice (Refs).

We isolated several SW480R and SW480A clones, which could be continuously grown for at least 20 passages without conversion of phenotypes. SW480R and SW480Adh cells differed by the levels of Prox-1, as determined by Northern and Western blotting (Fig. 3H-I). We compared

the gene expression profiles of SW480R and SW480A cells using oligonucleotide microarrays containing 22,000 annotated human genes, and identified about 1,000 genes whose expression differed by more than fourfold between these two cell types (Table I). A striking difference was observed in the expression of cytoskeletal and cell adhesion proteins. In agreement with their decreased adhesion and round cell shape, the SW480R cells lacked many components of the actin, intermediate filament and microtubule networks, such as gelsolin, filamins A and B, ezrin, moesin, vimentin, various integrins and tubulins (**Fig. 4A**, Table I and data not shown). These cells expressed higher levels of the protooncogenes c-myc and c-met, as well as the receptor tyrosine kinase FGFR-4, which has been associated with malignant transformation (Ullrich, pancreas), and low levels of the tumor suppressor p21Cip1 (Fig. 4B). In addition, all three tissue inhibitors of matrix metalloproteinases were absent from the SW480R cells, which may further account for their increased tumor growth *in vivo*. In contrast, the SW480A cells expressed higher levels of the chemokine receptor CXCR4, which is expressed in the normal colonic epithelium (Ref). In summary, the gene expression profile of the SW480R cells correlates well with a highly aggressive transformed phenotype, whereas the SW480A cells display more differentiated features typical of cells in the colonic crypts.

#### *Prox-1 silencing in SW480R cells leads to a differentiated and quiescent phenotype*

In order to investigate whether Prox-1 plays role in the generation and maintenance of the highly transformed phenotype, we suppressed Prox-1 mRNA and protein in the SW480R cells using Prox-1 targeting siRNA (**Fig. 5A**). Prox-1 siRNA-transfected cells but not the untransfected or GFP siRNA transfected cells underwent a morphological change, which became visible already at 72 h and persisted for up to 20 days after the transfection. The Prox-1 siRNA transfected cells become first more elongated and displayed extensive membrane ruffling. Later on, they started to spread on the plate and an number of increased actin stress fibers could be visualized by

phalloidine staining (Fig. 4B). We are planning to confirm the growth arrest and absence of apoptotic changes in Prox1 si transfected cells using FACS analysis. To verify the specificity of Prox1 silencing, we are currently repeating the analysis using a second set of siRNAs directed against a 5' region of Prox1 mRNA.

Since the siRNA transfection efficiency in our experiment was close to 100%, we analyzed the changes in the expression profiles of the SW480R and SW480A cells 120 and 240 h posttransfection, when the morphological changes were apparent. We identified only 29 down-regulated and 120 upregulated genes in Prox1 siRNA versus GFP siRNA transfected cells (Table II). 41% of these genes were differentially expressed between the SW480R and SW480A cells, suggesting that Prox-1 at least partially determines the phenotype of SW480R cells. Interestingly, the ablation of Prox-1 led to upregulation of a number of known epithelial markers, such as annexin A1, CRPB2, S100A3 and EMP1, along with the increase in cell adhesion molecules OB-cadherin and integrins beta7, beta5 and alpha 1. In line with the observed growth arrest we also observed the decrease in c-myc and a strong increase of CDK inhibitor p21Cip1 (Fig 4C).

Since anchorage-independent growth is one of the hallmarks of the malignant transformation we are planning to study whether the suppression of Prox1 will affect the ability of SW480R cells to grow in soft agar. For this purpose we will transfect SW480R cells with Prox1 siRNA or GFP siRNA, plate them at different densities in soft agar, and will score the number of colonies after two weeks.

To study the effects of Prox1 suppression in vivo, we will use SW480A cells stably overexpressing Prox1 or SW480R cells transfected with Prox1 si RNA to implant into nude mice. Rate of tumor growth, differentiation status and the gene expression using Affymetrix microarrays will be investigated.



*Ablation of Prox-1 leads to differentiation through up-regulation of Notch signaling in the SW480R cells.*

Notch signaling has been shown to be essential for the generation of cell lineages in the crypts of the mouse small intestine ( ). High levels of Notch are thought to suppress the expression of the basic helix-loop-helix transcription factor Math1 via the induction of the transcriptional repressor Hes1, which will lead to the differentiation of progenitor cells into enterocytes ( ). Conversely, high levels of Math1 results in the differentiation towards the neuroendocrine, Goblet and Paneth cell types in the small intestine. Among Notch signaling components, Notch2 and Hes1 levels are higher in SW480A cells in comparison with the SW480R cells, suggesting that this pathway is functionally active in these cells. Surprisingly, suppression of Prox1 resulted in up-regulation of the Notch ligand Jagged1 and in increased expression of transcription factor Hey2, a direct target of the Notch pathway, previously shown to be essential for cardiovascular development in mice and in zebrafish (Fig. 5C). In line with these findings, we also observed up-regulation of Notch-responsive reporter CBF1-luc in SW480R cells transfected with Prox1 siRNAs (Figure will be included). Conversely, overexpression of Prox1 in SW480A cells resulted in downregulation of Notch2 and jagged1 mRNA (Affymetrix results only, not confirmed by Northern).

In order to confirm the role of Notch in the regulation of SW480R cell growth and differentiation, we are planning to overexpress constitutively active Notch 1,2 and 3 intracellular domains or notch ligand jagged1 using recombinant adenoviruses and study their effects on the gene expression profile. In particular, we are interested in the regulation by Notch of cell cycle inhibitor p21Cip1. P21 Cip1 was shown to be involved in the regulation of growth arrest and differentiation in many cells types. Activation of TCF-4/b-catenin pathway in colon cancer is thought to suppress the expression of p21Cip1 via induction of c-myc. Interestingly, Notch1 intracellular domain was shown to induce the expression of p21 Cip1 in keratinocytes, and

ablation of Notch 1 in skin results in enhanced proliferation and increased susceptibility to chemically induced carcinogenesis (ref).

#### *Regulation of prostaglandin biosynthesis by Prox-1*

COX-2 is a key enzyme involved in the conversion of arachidonic acid into the prostaglandin precursors PGG<sub>2</sub> and PGH<sub>2</sub>, which are further transformed into biologically active prostaglandins by the action of corresponding synthases. Prostaglandins acts through binding to the G-protein coupled prostanoid receptors and they are rapidly inactivated by the action of 15-prostaglandin dehydrogenase (15-PGDH). COX-2 is overexpressed in the majority of colorectal cancers and in about half of colonic adenomas, suggesting that the increased PG production is important for tumor growth. In support of this view, treatment with NSAIDs, which acts as inhibitors of COX-2, significantly reduces the risk of developing colon cancer (ref).

We found that suppression of Prox-1 in SW480R cells resulted in the up-regulation of the expression of 15-PGDH and downregulation of prostaglandin D<sub>2</sub> synthase, whereas overexpression of Prox-1 in SW480F cells down-regulated 15-PGDH and up-regulated PGD<sub>2</sub> synthase (Affymetrix results only, not confirmed by Northern). These data suggest that Prox1 may be important for the control of the balance of the total PG production in tumor cells, i.e. in the presence of Prox1 decreased expression of 15-PGDH will result in higher net amounts of biologically active prostaglandins and enhanced tumor growth. Because SW480 cells do not express COX-2, we are planning to study the effects of Prox1 on prostanoid biosynthesis in the SW480F cells stably transfected with COX-2 or in the cell line which is known to express this enzyme, such as HCA-7. Cells will be infected with AdProx1 or the control AdGFP and the amount of biologically active PGE<sub>2</sub>, and total amount of metabolized PGE<sub>2</sub> will be determined by ELISA. If overexpression of Prox1 increases levels of the biologically active PGE<sub>2</sub> in vitro, we will further study the link between Prox1 overexpression and prostanoid biosynthesis in vivo.

In particular we will study the effects of 15-PGDH overexpression or treatment with 15-PGDH inhibitor on growth of Prox-1 expressing or control xenografts in nude mice.

## Discussion

Transcription factor Prox-1 is expressed in a large number of tissues during embryonic development, including lens fiber cells, subpopulation of neurons in brains and neural tube, skeletal muscle, heart, liver, pancreas and lymphatic endothelial cells. Targeted inactivation of Prox-1 results in the defects of eye development because of the failure of lens fiber cells to elongate (ref). In addition, Prox-1 deficient embryos lack lymphatic vasculature, while the blood vessel development is not affected (Ref). Prox-1 is also necessary for the migration of hepatocytes during liver development (ref). Recently, other and we have demonstrated the essential role of Prox-1 in the establishment of lymphatic endothelial phenotype. Overexpression of Prox-1 in blood endothelial cells, where it is otherwise absent, leads to the increased expression of lymphatic endothelial markers and to the suppression of the genes characteristic for the blood endothelial lineage (Refs.).

Our current study demonstrate for the first time the importance of Prox-1 in cancer. Prox-1 is overexpressed in 68% of colorectal malignancies as well as in 90% of the premalignant lesions, suggesting that it may be important for the disease progression. In normal colonic epithelium, Prox-1 expression is restricted to two cell types, neuroendocrine cells and non-proliferating cells at the very base of colonic crypts, a location that corresponds to the stem cell compartment. The expression pattern of Prox-1 suggest that it may be involved in the establishment of neuroendocrine cell fate as well as of the stem cell phenotype. We tested the this hypothesis by suppressing Prox-1 in a subclone of highly malignant subtype of colon adenocarcinoma cells SW480R. The ablation of Prox-1 results in cell growth arrest, increased expression of epithelial markers, and up-regulation of cell cycle inhibitor p21cip1, which was also shown to be important for the differentiation of intestinal epithelia (Ref), and the increased expression of the component of Notch signaling pathway. Unexpectedly, this phenotype persisted for up to two weeks after the

transfection, demonstrating the profound changes in the transcriptional program induced in the absence of Prox-1. We hypothesize that Prox1 may be involved in the maintenance of undifferentiated state of colonic intestinal stem cells, and that overexpression of Prox1 in cancer cells and resulting inhibition of Notch signaling pathway may lead to the de-differentiation frequently observed upon malignant transformation via. Our findings also suggest that that suppression of Prox1 or activation of Notch signaling in the tumor cells may prove beneficial for the treatment of colon cancer as well as of premalignant lesions of colon.

## **Methods**

*Antibodies, immunohistochemical and immunofluorescent staining.*

*RNA isolation, Northern blotting*

Total RNA was isolated and DNaseI treated in RNeasy columns (Qiagen). For Northern analysis, the blots were hybridized with  $^{32}\text{P}$ -labelled probes in Ultrahyb solution (Ambion).

## Figures

**Figure 1** Prox-1 mRNA is elevated in colorectal tumors. Cancer profiling array was hybridized to probes for Prox-1 (A) and lymphatic endothelial marker LYVE-1 (B).

**Figure 2.** Expression pattern of Prox1 in colon cancer and normal colon. Frozen sections of colon adenocarcinomas (A-C) or the corresponding normal tissues (D-F) were stained for Prox1. Double immunofluorescent staining for Prox-1 (K,M) and neuroendocrine marker chromogranin A (J) or proliferation marker Ki-67 (N) and merged images (O, L). Nuclei in (I) were visualized with Hoechst 33342.

**Figure 3.** Prox-1 is expressed in SW480R but not SW480Adh cells. (A) SW480 cells express Prox1. BEC, blood endothelial cells, LEC, lymphatic endothelial cells, served as negative and positive controls. (B-F) Prox1 expressing cells (green) are round and weakly adhesive. To visualize cell shape cells were double stained for F-actin marker phalloidin (B) or  $\beta$ -catenin (E). (G,I). Expression of Prox-1 in isolated subclones of SW480R and SW480ADH cells detected by Northern and Western blotting. Hybridization for GAPDH was used as loading control.

**Figure 4.** Examples of genes differentially expressed between SW480R and SW480ADH cells. (A) SW480 cells were stained for intermediate filament protein vimentin (red) and Prox1 (green). (B). Northern blotting and hybridization for the indicated transcripts. Hybridization for GAPDH was used as loading control.

**Figure 5.** Suppression of Prox-1 in SW480R cells results in morphological changes, growth arrest and activates Notch signaling pathway. (A) Transfection with Prox1 si RNAs but not GFP si RNAs suppresses Prox-1 protein. Immunofluorescent staining for Prox1(green), nuclei

are visualized with Hoechst 33342 (blue). (B) Changes in actin cytoskeleton in Prox1 si RNA transfected cells at 120 and 240 h. (C) Examples of genes differentially expressed in SW480R Prox si RNA transfected cells versus non-transfected and GFPsi transfected controls. 120h and 240h time points are shown. Northern blotting and hybridization for the indicated transcripts. (D) Prox-1 suppression up-regulates the activity of CBF1-luc reporter. SW480R cells were transfected with GFP si RNA or Prox si RNA and GFB1-luc or the control reporter plasmid.

## References



Table I. Examples of groups of genes differentially expressed in round versus adherent SW480 clones. Two round and two adherent clones were analysed.

Gene function and name	Gene symbol	Log <sub>2</sub> ratio, average	stdev
<b>1. Cytoskeleton and adhesion</b>			
collagen, type XIII, alpha 1	COL13A1	-5.6	0.9
fibronectin 1	FN1	-5.2	0.5
integrin, alpha 7	ITGA7	-4.3	0.3
vimentin	VIM	-4.1	0.6
filamin B, beta (actin binding protein 278)	FLNB	-3.8	0.7
integrin, beta 5	ITGB5	-3.6	0.5
tubulin, beta polypeptide	TUBB	-3.3	0.7
PTPL1-associated RhoGAP 1	PARG1	-3.0	0.5
collagen, type IX, alpha 3	COL9A3	-2.8	0.8
paralemmmin	PALM	-2.7	0.2
PDZ and LIM domain 1 (elfin)	PDLIM1	-2.7	0.2
cadherin 11, type 2, OB-cadherin (osteoblast)	CDH11	-2.6	0.7
myosin IC	MYO1C	-2.6	0.6
integrin, alpha 3	ITGA3	-2.6	0.4
discs, large (Drosophila) homolog 1	DLG1	-2.5	0.1
integrin, alphaV	ITGAV	-2.5	0.3
CDC42 effector protein (Rho GTPase binding) 3	CDC42EP3	-2.4	0.4
ephrin-B1	EFNB1	-2.3	0.4
FERM, RhoGEF (ARHGEF) and pleckstrin domain protein 1	FARP1	-2.3	0.4
myosin ID	MYO1D	-2.1	0.2
PDZ and LIM domain 2 (mystique)	PDLIM2	-2.1	0.4
tubulin beta-5	TUBB-5	-1.9	0.3
erythrocyte membrane protein band 4.1-like 1	EPB41L1	-1.9	0.1
gelsolin (amyloidosis, Finnish type)	GSN	-1.9	0.3
laminin, gamma 1 (formerly LAMB2)	LAMC1	-1.8	0.1
ras homolog gene family, member E	ARHE	-1.7	0.2
IQ motif containing GTPase activating protein 1	IQGAP1	-1.7	0.3
tight junction protein 1 (zona occludens 1)	TJP1	-1.7	0.4
catenin (cadherin-associated protein), alpha-like 1	CTNNAL1	-1.7	0.6
collagen, type XVIII, alpha 1 absent in colon adenocarcinomas	COL18A1	-1.6	0.1
filamin A, alpha (actin binding protein 280)	FLNA	-1.6	0.2
actin related protein 2/3 complex, subunit 1A, 41kDa	ARPC1A	-1.5	0.3
alpha integrin binding protein 63	AIBP63	-1.4	0.3
spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	SPTAN1	-1.4	0.2
villin 2 (ezrin)	VIL2	-1.4	0.3
actin related protein 2/3 complex, subunit 1B, 41kDa	ARPC1B	-1.3	0.1
plakophilin 4	PKP4	-1.3	0.3
ras homolog gene family, member C	ARHC	-1.1	0.1
moesin	MSN	-1.1	0.1
myristoylated alanine-rich protein kinase C substrate	MARCKS	-1.1	0.2
<b>2. Tumor growth and invasion</b>			
tissue inhibitor of metalloproteinase 2	TIMP2	-2.3	0.21
tissue inhibitor of metalloproteinase 3	TIMP3	-1.5	0.14

Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	-2.5?	?
tissue inhibitor of metalloproteinase 1	TIMP1	-1.5	0.4
met proto-oncogene (hepatocyte growth factor receptor)	MET	2.6	0.46
v-myc myelocytomatosis viral oncogene homolog	MYC	1.2	?
Fibroblast growth receptor 4	FGRFR4	3.9	0.76

### 3. Expressed in normal intestinal epithelium

CXCR4

Solute carrier 7

Table II. Genes regulated by Prox-1 in SW480R cells. Genes differentially expressed between SW480R and SW480ADH cells are shown in bold.

Genes down-regulated in the absence of Prox-1	Gene symbol	Log <sub>2</sub> ratio, average	stdev
Nebulette	NEBL	-2.0	0.4
<b>transforming growth factor, beta-induced, 68kDa</b>	<b>TGFB1</b>	<b>-1.9</b>	<b>0.1</b>
<b>trinucleotide repeat containing 9</b>	<b>TNRC9</b>	<b>-1.9</b>	<b>0.2</b>
insulin-like growth factor binding protein 3	IGFBP3	-1.6	0.0
calpain 1, (mu/I) large subunit	CAPN1	-1.5	0.3
<b>inhibitor of DNA binding 1</b>	<b>ID1</b>	<b>-1.5</b>	<b>0.3</b>
midkine (neurite growth-promoting factor 2)	MDK	-1.5	0.1
FK506 binding protein 11, 19 kDa	FKBP11	-1.4	0.1
caspase recruitment domain family, member 10	CARD10	-1.3	0.1
<b>inhibin, beta B (activin AB beta polypeptide)</b>	<b>INHBB</b>	<b>-1.3</b>	<b>0.2</b>
L1 cell adhesion molecule	L1CAM	-1.2	0.1
<b>glutathione peroxidase 2 (gastrointestinal)</b>	<b>GPX2</b>	<b>-1.2</b>	<b>0.0</b>
<b>eukaryotic translation elongation factor 1 alpha 2</b>	<b>EEF1A2</b>	<b>-1.2</b>	<b>0.2</b>
hypothetical protein FLJ11149	FLJ11149	-1.2	0.2
potassium voltage-gated channel, subfamily H (eag-related), member 2	KCNH2	-1.1	0.1
<b>KIAA0182 protein</b>	<b>KIAA0182</b>	<b>-1.1</b>	<b>0.0</b>
lectin, galactoside-binding, soluble, 1 (galectin 1)	LGALS1	-1.1	0.1
Homo sapiens cDNA FLJ41000 fis,		-1.1	0.3
<b>ephrin-B2</b>	<b>EFNB2</b>	<b>-1.1</b>	<b>0.1</b>
<b>v-myc myelocytomatosis viral oncogene homolog (avian)</b>	<b>MYC</b>	<b>-1.1</b>	0.1
S100 calcium binding protein A14	S100A14	-1.1	0.2
<b>Alpha one globin [Homo sapiens], mRNA sequence*</b>		<b>-1.1</b>	<b>0.1</b>
<b>hypothetical protein FLJ10986*</b>	<b>FLJ10986</b>	<b>-1.0</b>	<b>0.0</b>
<b>hypothetical protein FLJ11149</b>	<b>FLJ11149</b>	<b>-1</b>	<b>0.0</b>
<b>myelin transcription factor 1*</b>	<b>MYT1</b>	<b>-1.0</b>	<b>0.0</b>
nucleolar autoantigen (55kD) similar to rat synaptonemal complex protein*	SC65	-1.0	0.1
tumor necrosis factor receptor superfamily, member 6b, decoy	TNFRSF6B	-1.0	0.1
<b>jagged 2</b>	<b>JAG2</b>	<b>-1.0</b>	<b>0.1</b>
mitochondrial ribosomal protein S2	MRPS2	-1.0	0.1
<b>Total: 29 genes</b>			

Genes up-regulated in the absence of Prox1	Gene symbol	Log2 ratio. average	Stdev
<b>insulin-like growth factor binding protein 7*</b>	<b>IGFBP7</b>	<b>5.8</b>	<b>0.4</b>
chitinase 3-like 1 (cartilage glycoprotein-39)*	CHI3L1	5.3	0.8
<b>chemokine (C-X-C motif) receptor 4*</b>	<b>CXCR4</b>	<b>4.5</b>	<b>1.1</b>
semaphorin 3C*	SEMA3C	4.5	4.5
<b>cadherin 11, type 2, OB-cadherin (osteoblast)*</b>	<b>CDH11</b>	<b>3.8</b>	<b>0.3</b>
annexin A1	ANXA1	3.7	1.1
<b>hypothetical protein MGC10796*</b>	<b>MGC10796</b>	<b>3.3</b>	<b>0.4</b>
<b>CD44 antigen</b>	<b>CD44</b>	<b>2.6</b>	<b>1.1</b>
Homo sapiens clone 23785 mRNA sequence		2.9	0.4
<b>epithelial membrane protein 1*</b>	<b>EMP1</b>	<b>2.9</b>	<b>0.1</b>
<b>inhibitor of DNA binding 2, dominant negative helix-loop-helix protein*</b>	<b>ID2</b>	<b>2.8</b>	<b>0.1</b>
<b>Human HepG2 3' region cDNA, clone hmd1f06, mRNA sequence</b>		<b>2.8</b>	<b>0.3</b>
<b>tumor necrosis factor receptor superfamily, member *11b (osteoprotegerin)</b>	<b>TNFRSF11B</b>	<b>2.6</b>	<b>0.7</b>
likely homolog of mouse glucuronyl C5-epimerase*	GLCE	2.6	1.1
ribonuclease, RNase A family, 1 (pancreatic)*	RNASE1	2.6	0.1
<b>apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B*</b>	<b>APOBEC3B</b>	<b>2.5</b>	<b>0.1</b>
<b>hydroxyprostaglandin dehydrogenase 15-(NAD)*</b>	<b>HPGD</b>	<b>2.5</b>	<b>1.1</b>
NPD009 protein	NPD009	2.5	0.6
integrin, beta 7*	ITGB7	2.4	0.1
fibroblast growth factor 20*	FGF20	2.3	1.0
KIAA0455 gene product	KIAA0455	2.3	1.3
CAMP-specific phosphodiesterase 8B1 [Homo sapiens], mRNA sequence*		2.3	0.4
<b>ectodermal-neural cortex (with BTB-like domain)*</b>	<b>ENC1</b>	<b>2.3</b>	<b>0.2</b>
frizzled homolog 1 (Drosophila)*	FZD1	2.3	0.8
S100 calcium binding protein A3*	S100A3	2.2	0.6
zeta-chain (TCR) associated protein kinase 70kDa*	ZAP70	2.2	1.1
platelet derived growth factor C*	PDGFC	2.1	0.1
cystatin D *	CST5	2.1	0.3
<b>CCAAT/enhancer binding protein (C/EBP), delta</b>	<b>CEBPD</b>	<b>2.1</b>	<b>0.1</b>
<b>sorbin and SH3 domain containing 1</b>	<b>SORBS1</b>	<b>2.1</b>	<b>0.5</b>
metallothionein 2A	MT2A	2.0	0.6
RAS guanyl releasing protein 1 (calcium and DAG-regulated)	RASGRP1	2.0	0.4
<b>checkpoint suppressor 1</b>	<b>CHES1</b>	<b>2.0</b>	<b>0.4</b>
<b>chondroitin beta1,4 N-acetylgalactosaminyltransferase*</b>	<b>ChGn</b>	<b>2.0</b>	<b>0.4</b>
<b>filamin B, beta (actin binding protein 278)*</b>	<b>FLNB</b>	<b>2.0</b>	<b>0.4</b>
aldehyde dehydrogenase 1 family, member A2*	ALDH1A2	2.0	0.6
jagged 1 (Alagille syndrome)	JAG1	2.0	0.1
<b>A kinase (PRKA) anchor protein (gravin) 12*</b>	<b>AKAP12</b>	<b>1.9</b>	<b>0.1</b>

metallothionein 1X*	MT1X	1.9	0.8
<b>creatine kinase, mitochondrial 2 (sarcomeric)</b>	<b>CKMT2</b>	<b>1.8</b>	<b>0.6</b>
serum-inducible kinase	SNK	1.8	0.1
<b>CGI-130 protein</b>	<b>CGI-130</b>	<b>1.8</b>	<b>0.1</b>
guanine nucleotide binding protein (G protein), alpha	GNAI1	1.8	0.4
inhibiting activity polypeptide 1	RNTRE	1.7	0.4
related to the N terminus of tre*			
solute carrier family 12 (sodium/potassium/chloride	SLC12A2	1.7	0.3
transporters), member 2		1.7	1.0
Human clone 23612 mRNA sequence	ASB4	1.7	0.8
ankyrin repeat and SOCS box-containing 4			
apolipoprotein B mRNA editing enzyme, catalytic	APOBEC3C	1.7	0.1
polypeptide-like 3C	<b>CRABP2</b>	<b>1.7</b>	<b>0.1</b>
<b>cellular retinoic acid binding protein 2*</b>	<b>KIAA0657</b>	<b>1.7</b>	<b>1.1</b>
<b>KIAA0657 protein*</b>			
phosphodiesterase 4D, cAMP-specific	PDE4D	1.7	0.1
(phosphodiesterase E3 dunce homolog, Drosophila)	AUTS2	1.6	0.4
autism susceptibility candidate 2	HEY2	1.6	0.0
hairy/enhancer-of-split related with YRPW motif 2*	<b>IER5</b>	<b>1.6</b>	<b>0.1</b>
<b>immediate early response 5</b>	SMURF2	1.6	0.4
E3 ubiquitin ligase SMURF2	<b>ARL7</b>	<b>1.6</b>	<b>1.0</b>
<b>ADP-ribosylation factor-like 7*</b>	<b>RIN2</b>	<b>1.6</b>	<b>0.4</b>
<b>Ras and Rab interactor 2*</b>	GS3955	1.6	0.5
GS3955 protein	MT1L	1.5	0.6
metallothionein 1L	GRM8	1.5	0.2
glutamate receptor, metabotropic 8	KL	1.5	0.1
klotho	CALML3	1.4	0.6
calmodulin-like 3	ITGA1	1.4	0.1
integrin, alpha 1	<b>LEF1</b>	<b>1.4</b>	<b>0.4</b>
<b>lymphoid enhancer-binding factor 1</b>	EVA1	1.4	0.1
epithelial V-like antigen 1	<b>LBH</b>	<b>1.4</b>	<b>0.1</b>
<b>likely ortholog of mouse limb-bud and heart gene*</b>	LOC51141	1.4	0.2
insulin induced protein 2	PTCH	1.4	0.1
patched homolog (Drosophila)	<b>CKLF6</b>	<b>1.3</b>	<b>0.3</b>
<b>chemokine-like factor super family 6</b>	LHFP	1.3	0.4
lipoma HMGIC fusion partner	TGFA	1.3	0.4
transforming growth factor, alpha			
Homo sapiens mRNA; cDNA DKFZp762M127 (from		1.3	0.6
clone DKFZp762M127), mRNA sequence	CCNI	1.3	0.1
cyclin I	<b>HAS2</b>	<b>1.3</b>	<b>0.5</b>
<b>hyaluronan synthase 2</b>	<b>IQGAP1</b>	<b>1.3</b>	<b>0.5</b>
<b>IQ motif containing GTPase activating protein 1</b>	ZNF216	1.3	0.2
zinc finger protein 216		1.3	0.2
cDNA DKFZp564O0122	AHR	1.2	0.6
aryl hydrocarbon receptor	NET1	1.2	0.1
neuroepithelial cell transforming gene 1	SC4MOL	1.2	0.1
sterol-C4-methyl oxidase-like	TUBA3	1.2	0.1
tubulin, alpha 3	BIGM103	1.2	0.0
BCG-induced gene in monocytes, clone 103	CTSB	1.2	0.0
cathepsin B			

keratin 6A	KRT6A	1.2	0.4
paraoxonase 2	PON2	1.2	0.4
suppressor of cytokine signaling 5	SOCS5	1.2	0.4
KIAA0877 protein	KIAA0877	1.2	0.2
<b>propionyl Coenzyme A carboxylase alpha</b>	<b>PCCA</b>	<b>1.2</b>	<b>0.2</b>
<b>solute carrier family 2</b>	<b>SLC2A3</b>	<b>1.2</b>	<b>0.1</b>
<b>solute carrier family 7</b>	<b>SLC7A8</b>	<b>1.2</b>	<b>0.1</b>
Homo sapiens mRNA; cDNA DKFZp762M127		1.2	0.1
aryl hydrocarbon receptor nuclear translocator-like	ARNTL	1.1	0.3
DnaJ (Hsp40) homolog, subfamily B, member 6	DNAJB6	1.1	0.3
hypothetical protein FLJ21276	FLJ21276	1.1	0.1
<b>integrin, beta 5</b>	<b>ITGB5</b>	<b>1.1</b>	<b>0.1</b>
PTK7 protein tyrosine kinase 7	PTK7	1.1	0.3
transforming growth factor, beta receptor II	TGFBR2	1.1	0.1
Homo sapiens cDNA FLJ25134 fis		1.1	0.0
	<b>DKFZP564A2</b>		
<b>DKFZP564A2416 protein</b>	<b>416</b>	<b>1.1</b>	<b>0.1</b>
<b>dual specificity phosphatase 6</b>	<b>DUSP6</b>	<b>1.1</b>	<b>0.4</b>
midline 1 (Opitz/BBB syndrome)	MID1	1.1	0.1
membrane protein, palmitoylated 1, 55kDa	MPP1	1.1	0.1
<b>LIM domain protein</b>	<b>RIL</b>	<b>1.1</b>	<b>0.1</b>
<b>SH3-domain binding protein 5 (BTK-associated)</b>	<b>SH3BP5</b>	<b>1.1</b>	<b>0.1</b>
SIPL protein	SIPL	1.1	0.1
<b>tumor protein D52-like 1</b>	<b>TPD52L1</b>	<b>1.1</b>	<b>0.4</b>
3-hydroxy-3-methylglutaryl-Coenzyme A reductase	HMGCR	1.0	0.1
<b>homeo box B7</b>	<b>HOXB7</b>	<b>1.0</b>	<b>0.1</b>
HIV-1 Tat interactive protein 2, 30kDa	HTATIP2	1.0	0.1
<b>insulin receptor substrate 2</b>	<b>IRS2</b>	<b>1.0</b>	<b>0.1</b>
<b>tubulin beta-5</b>	<b>TUBB-5</b>	<b>1.0</b>	<b>0.0</b>
apoptosis antagonizing transcription factor	AATF	1.0	0.1
E2F transcription factor 3	E2F3	1.0	0.1
hypothetical protein FLJ12542	FLJ12542	1.0	0.1
phafin 2	FLJ13187	1.0	0.1
<b>proline 4-hydroxylase</b>	<b>P4HA2</b>	<b>1.0</b>	<b>0.1</b>
<b>Homo sapiens G21VN02 mRNA, mRNA sequence</b>		<b>1.0</b>	<b>0.1</b>